(i.e., A-N interdomain distance). FRET results were compared to distance predictions from x-ray crystallography. We propose a structural mechanism for ligand activation of SERCA. Acknowledgments: Spectroscopy was performed in the Biophysical Spectroscopy Center at the University of Minnesota, with assistance from Fluorescence Innovations, Inc. (Gregory Gillispie, President). This work was funded by NIH grants to DDT (R01 GM27906, P30 AR0507220, T32 AR007612).

**1529-Pos Board B421**

**Screening for SERCA Activators using a High-throughput Time-Resolved FRET Assay**

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We used a prototype time-resolved fluorescence lifetime microplate reader to carry out a high-throughput screen designed to identify compounds that interact with the sarcoplasmic reticulum calcium ATPase (SERCA). SERCA is essential for the Ca homeostasis in many cell types. Insufficient SERCA activity leads to cardiovascular disease, muscular dystrophy, skin disease, and diabetes. Our goal is to discover activators of SERCA that can be developed into drugs to treat diseases in which Ca transport is deficient. The fluorescence lifetime plate reader was made possible by our recent development of fast time-resolved fluorescence by direct waveform recording, which achieves 105 higher throughput than the conventional single-photon counting technology. using this plate reader, we detected fluorescence resonance energy transfer (FRET) between IAEDANS-labeled SERCA and nucleotide analog TNPADP in native sarcoplasmic reticulum membranes. This assay was designed to detect compounds that interact with SERCA and modify either the enzyme's structure or the binding affinity of TNP-ADP. Initial hit compounds were further analyzed in functional assays. Upon screening a small (1300 compound) library, we determined that the time-resolved microplate reader has at least 10x higher precision than a conventional intensity-based microplate reader, raising the quality index (z') of our assay from marginal, in the intensity reader, to excellent. A 384-well plate is read with high precision in 2 min, which allows screening of thousands of compounds/day. An important advantage of the time-resolved fluorescence measurement is that it provides detailed structural information, thus enabling discovery of multiple classes of compounds during the primary screen.

**1530-Pos Board B422**

**In-Cell FRET as a Tool to Develop SERCA Activators for Drug or Gene Therapy**

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We are using in-cell FRET methods (Photobleaching, FLIM, time-resolved FRET in a plate reader) to study the relationships among structure, dynamics, and function of the sarcoplasmic reticulum Ca-ATPase (SERCA) and its cardiac regulator phospholamban (PLB), with the goal of designing activators of SERCA for treatment of heart failure (HF) and muscular dystrophy (MD). $Ca^{2+}$ drives muscle contraction, and relaxation is accomplished by the sequestration of $Ca^{2+}$ by the sarcoplasmic reticulum Ca-ATPase (SERCA), which is inhibited by phospholamban at submicromolar [$Ca^{2+}$] in cardiac muscle. SERCA activity is frequently reduced in HF, and many current therapeutic strategies aim to increase cardiac $Ca^{2+}$ cycling activity. We are designing LOF-PLB mutants ($PLB_M$) that can compete with WT-PLB ($PLB_W$) and thus relieve SERCA inhibition. Our ideal mutant is partial loss-of-function, binds tightly to SERCA2a, and remains phosphorylatable via β-adrenergic pathways. The effects of $PLB_M$ on Ca-ATPase activity and FRET are measured to determine the mutant's ability to compete with $PLB_W$, both physically and functionally. Optimal LOF mutants are being tested in a rat model of heart failure.

In addition to this SERCA-PLB FRET competition assay, we are using SERCA2a labelled with two different fluorescent probes (GFP and RFP) to screen for small molecule activators of SERCA in living cells. Time-resolved FRET measurements of RFP-GFP-SERCA2a in 96- or 384-well plates have shown the extraordinary precision and sensitivity necessary to probe SERCA's structure-function relationship. New drug screening efforts are active and small molecules from previous screens are being developed as promising drugs for HF therapy (collaboration with Celladon, Inc.). Many of these drugs are direct SERCA activators and are being investigated for their ability to rescue the muscular dystrophy phenotype in dystrophic mice.

**1531-Pos Board B423**

**EPR Detects Changes in the Transmembrane Region of the SERCA-Phospholamban Complex upon Ser16 Phosphorylation**

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We have used site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy to investigate the effects of Ser16 phosphorylation on phospholamban's interaction with the sarcoplasmic reticulum Ca-ATPase (SERCA). Muscle contraction is signaled by the release of Ca from the sarcoplasmic reticulum (SR), while SERCA mediates muscle relaxation by actively pumping Ca back into the SR lumen. Cardiac SERCA is regulated by phospholamban (PLB), a single-pass transmembrane protein that inhibits the Ca pump unless phosphorylated at Ser16.

We have shown that Ser16 phosphorylation does not dissociate PLB from SERCA to relieve inhibition, but instead induces a structural change in PLB's transmembrane (TM) helix that restores SERCA Ca sensitivity. To characterize this change, we have attached spin labels along the TM helix and performed EPR accessibility measurements to determine the effects of Ser16 phosphorylation on TM helix topology within the regulatory complex. Our results show that upon phosphorylation, the TM helix undergoes a vertical shift that could break inhibitory interactions between SERCA and PLB. Complementary ongoing studies employ these spin-labeled PLB constructs to determine changes in (a) the orientation of PLB and (b) distances to probes on SERCA, with the goal of obtaining a detailed structural model for the regulatory transitions within the SERCA-PLB complex.

**1532-Pos Board B424**

**Orientation of Phospholamban in Lipid Bicelles Detected by Electron Paramagnetic Resonance**

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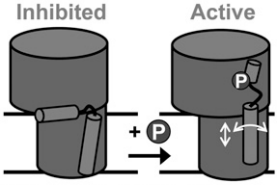
We have used electron paramagnetic resonance (EPR) to probe the structural dynamics of the integral membrane protein phospholamban (PLB), as a function of phosphorylation and addition of its regulatory target, the sarcoplasmic reticulum calcium ATPase (SERCA). We found previously that PLB remains bound to SERCA after phosphorylation, suggesting that a structural transition within the SERCA-PLB complex is responsible for relief of inhibition. Our current goal is to elucidate this mechanism through orientation and accessibility EPR, in order to support rational design of therapies to improve calcium transport in muscle cells. We used the monomeric mutant AFA-PLB, with the rigid electron spin label TOAC incorporated within the transmembrane domain, and reconstituted the protein in lipid bicelles and vesicles. EPR showed that the accessibilities of PLB spin labels to paramagnetic relaxation agents changed upon phosphorylation, indicating vertical (parallel to the membrane normal) movement of PLB. EPR on aligned bicelles showed that PLB changes its tilt relative to the membrane upon phosphorylation. Results will also be reported on structural changes in the presence of SERCA. This work was funded by grants from NIH (R01 GM27906 and T32 AR007612).



PLB binds to SERCA forming an inhibited complex. This inhibition is relieved upon PLB phosphorylation by a structural change, which may include vertical and tilting motions.

**1533-Pos Board B425**

**Probing Cardiac Membrane Proteins with Fluorescence Resonance Energy Transfer**

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We have used time-resolved fluorescence resonance energy transfer (TR-FRET) to study the structural basis of regulation of sarcoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) by a single-pass transmembrane protein, phospholamban (PLB). The most prominent feature of heart failure is calcium mishandling, which is largely due to impaired activity of sarcoplasmic reticulum calcium ATPase (SERCA), which actively transports $Ca^{2+}$ from cytosol into the SR to relax the muscle. Unphosphorylated phospholamban inhibits SERCA by decreasing its apparent $Ca^{2+}$ affinity. This inhibition can be relieved by either micromolar $Ca^{2+}$ or by phosphorylation of PLB. The structural basis for relief of inhibition remains controversial. Cross-linking studies support the canonical dissociation model. However, recent EPR, NMR and FRET studies support the subunit model, in which subtle structural rearrangements are required to